Collagen α5 and α2(IV) chain coexpression: Analysis of skin biopsies of Alport patients

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Alport syndrome is a collagen type IV disease caused by mutations in the COL4A5 gene with the X-linked form being most prevalent. The resultant α5(IV) collagen chain is a component of the glomerular and skin basement membranes (SBMs). Immunofluorescent determination of the α5(IV) chain in skin biopsies is the procedure of choice to identify patients. In 30% of patients, however, the mutant protein is still found in the SBM resulting in a normal staining pattern. In order to minimize or eliminate false results, we compared the distribution of the α2(IV) chain (another SBM component) and the α5(IV) chain by standard double label immunofluorescence (IF) and by confocal laser scanning microcopy. The study was performed on 55 skin biopsies of patients suspected of Alports and five normal control specimens. In normal skin, IF showed the classical linear pattern for both collagens along the basement membrane. Additionally, decreased α5(IV) was found in the bottom of the dermal papillary basement membrane. Confocal analysis confirmed the results and show α5(IV) focal interruptions. In suspected patients, both techniques showed the same rate of abnormal α5(IV) expression: segmental in women and absent in men. Our results show a physiological variation of α5(IV) location with focal interruptions and decreased expression in the bottom of the dermal basement membrane. Comparison of α5(IV) with α2(IV) expression is simple and eliminates technical artifacts.

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Alport syndrome (AS) is a type IV collagen disease.[1] The X-linked form of the AS is the most prevalent transmission (85%), and is caused by mutations in COL4A5.[2] Approximately, 15% of AS are caused by COL4A3 and COL4A4 mutations with an autosomal recessive or rarely dominant pattern of inheritance.[1,3,4] The α1 chain of collagen IV (IV), α2(IV), α3(IV), α4(IV), and α5(IV) chains are components of glomerular basement membrane.[5] Skin basement membrane (SBM) is composed of α1(IV), α2(IV), α5(IV), and α6(IV), but not α3(IV) and α4(IV), rendering skin analysis uninformative in autosomal AS. In most X-linked AS patients, immunofluorescence (IF) analysis of the skin biopsy shows lack of α5(IV) chain, making skin biopsy a procedure of choice to identified X-linked AS patients.[6–9] However, in 30–40% of patients with proven COL4A5 mutations, immunohistochemical staining for α5(IV) is normal, suggesting that the mutation had not prevented incorporation of the α5(IV) chain into glomerular and SBM.[10–13]

Recently, it was suggested that confocal laser staining microscopy (CLSM) skin analysis is more precise, allowing identification of an irregular distribution rather than the absence of the α5(IV) chain in male and female cases with COL4A5 mutations.[14]

The aim of the study was to more precisely differentiate between physiological and abnormal distribution of α5(IV) chain in SBM of AS patients. For this purpose, we used dual staining for α2(IV), which is not affected in AS, and α5(IV). Furthermore, we studied all biopsies with standard and CLSM.

RESULTS

In normal skin, standard double direct IF showed an uninterrupted linear pattern of α2(IV) and α5(IV) along the SBM (Figure 1a–c). Unexpectedly, we observed decreased or absence of α5(IV) expression in the bottom of dermal papillary BM, contrary to α2(IV), which was always strongly expressed (Figure 1d–f).

CLSM gave the same results than standard IF (not shown). In addition, the high resolution image by CSLM revealed that α5(IV) could be focally absent in normal skin (two out of

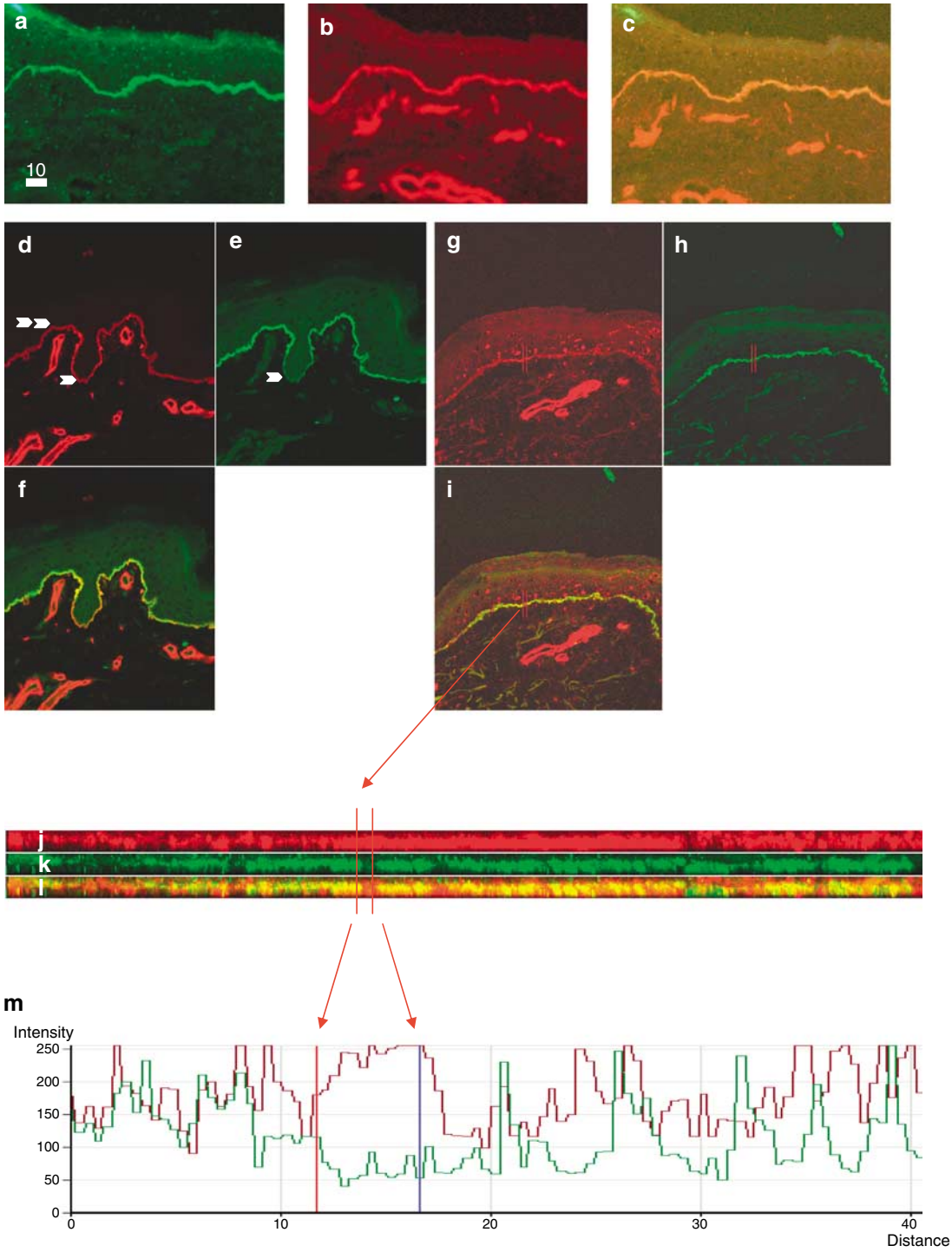

Figure 1 | Expression of α5(IV) in normal skin. (**a–c**) Normal skin (20 × /0.50 standard dual immunostaining). Expression of α5(IV) in green (**a**) and α2(IV) in red (**b**). (**c**) Merge Bar 10 μm. (**d–f**) Normal skin in papillary zone (10 × standard dual immunostaining). (**d**) α2(IV) in red was expressed along EBM in the upper (double arrow) and the bottom (arrow) of the dermal papillary basement membrane, and strongly on capillary wall. (**e**) α5(IV) in green was expressed along EBM but decreased in the bottom of dermal papillary basement membrane (arrow) (**f**) merge. (**g–m**) Focal interruption of α5(IV) (10 × CLSM). (**g**) Continuous positive staining of α2(IV), (**h**) but focal absence of α5(IV) in normal skin (red lines), (**i**) merge. (**j**) Z projection of α2(IV), (**k**) projection of α5(IV), and (**l**) merge of Z projection. (**m**) Quantitative analysis using imaging analysis software of an LSM510 system: focal negative segment with α5(IV) (green curve) is included between 12 and 17 μm distance (red and blue lines) compared with α2(IV) (red curve).

five) in segments $<5\,\mu$m long, contrary to α2(IV), which remained positive (Figure 1g–i). These small foci of negative staining were even more obvious when quantitative analysis of fluorescence was performed (Figure 1j–m).

In male patients suspected of having X-linked AS, 11/20 biopsies (55%) showed α2 and α5(IV) normal expression with standard IF. Using CSLM, no defect potentially missed by double standard IF could be observed in these 11 patients. In nine cases, α5(IV) was totally absent (Figure 2a), whereas, the α2(IV) staining was normal (Figure 2b), with both methods.

Segmental α5(IV) distribution was never observed in any of the male patients, even with CSLM.

In female patients, 18/ 35 biopsies (51%) showed normal expression of both chains with standard IF. Again, CSLM did not identify abnormal staining in these18 female patients. Sixteen biopsies showed a segmental α5(IV) (Figure 2c and d) expression with a normal α2(IV) staining. Both standard IF and CSLM disclosed the same pattern.

In one case, α5(IV) was totally absent with normal α2(IV) distribution using both standard IF and CSLM.

In addition, among the 45 patients with continuous (11 male subjects) or mosaic (34 female subjects) positivity of α5(IV), CSLM revealed that in 7/45, α5(IV) was clearly absent in small foci ($<5\mu$m long) along the SBM, contrary to α2(IV), which was always strongly expressed. This pattern was similar to that observed in normal skin (cf above).

In two cases, direct comparison of α5(IV) with α2(IV) (inner control) revealed technical artifact. In those cases, the two chains were negative because of the absence of antibodies penetration on tissue sample (folds in the skin, Figure 3a–d or air bubble Figure 3e–g).

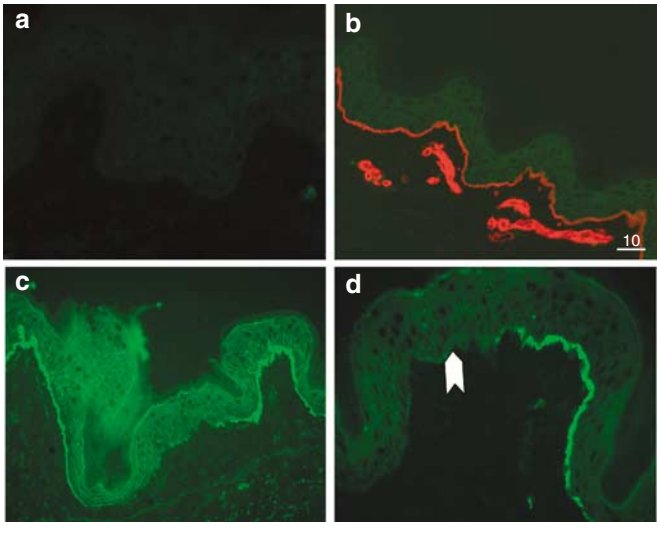

Figure 2 | Expression of α5(IV) in the skin of patients meeting criteria for Alport. Male patient with AS (10 × standard dual immunostaining): (**a**) absence of α5(IV) and (**b**) normal expression of α2(IV). Female patient with AS (standard dual immunostaining): (**c**) segmental expression of α5(IV) (10 × 0.50) (**d**, arrow) especially in the upper part of the papillary dermis BM (20 × /0.50).

When skin section was not perpendicular to SBM, we observed a coarsely granular distribution of α5(IV), which corresponds to a decreased signal with both methods (Figure 3h). A similar decreased signal was observed with α2(IV) (Figure 3i), excluding an abnormal expression of α5(IV).

DISCUSSION

It was recently described that CSLM analysis of skin biopsies could provide a more sensitive approach to diagnose AS when normal α5(IV) staining is observed by conventional IF, eliminating 'false-negative' results.[14] The aim of the study was to compare the two methods, but not to appreciate the impact of each method in term of sensibility and specificity. For this reason, we included all the biopsy received for 'suspected AS patients' and not only biopsy of patients with unequivocal clinical criteria of AS.

We applied standard IF and confocal analysis to double direct IF in skin biopsies with a solution containing antibodies against α5(IV) and α2(IV) chains (Figure 1). The most important finding we observed with both methods was a decreased or a disappearance of α5(IV), but not α2(IV), along the bottom of dermal papillary BM in normal skin. This physiological variation of α5(IV) expression is described for the first time and could be related to local BM specialization, as it was suggested for hair growth cycle.[15] Thus, analysis of Alport female skin biopsies has to be interpreted with caution. Abnormal expression of α5(IV) can be ascertained only when the negative zone is distant from the bottom of dermal papillary BM and compared to α2(IV). In male patients, the expression of α5(IV) was normal or totally absent, and segmental expression was never found, even with CLSM.

In addition, we noted focal interruptions of α5(IV) ($<5\,\mu$m long, quantified by the standard imaging analysis software of an LSM 510 system) in both several control and AS skin biopsies, without α2(IV) abnormality, excluding a technical artifact. We, thus, interpret this pattern as a physiological variation of α5(IV) distribution contrary to Muda *et al.*[14]

Conventional double IF using combined α5(IV) and α2(IV) antibodies is fast and simple: (1) no antigen demasking by urea denaturation is needed, (2) only one section is necessary, (3) direct comparison of α5(IV) with α2(IV) (inner control) avoid misinterpretation related to technical artifact, (4) in our hands, CSLM results were identical to double IF and did not ascertain the existence of 'false-negative results' obtained with conventional IF.

In conclusion, skin biopsy is now the first choice in diagnosing AS. When excluding technical artefacts and physiological variations described herein, it is absolutely specific both for AS and for the X-linked mode of inheritance. However, it remains uninformative in a substantial proportion of patients when normal expression of α5(IV) is observed. Future developments in DNA analysis may circumvent these drawbacks.

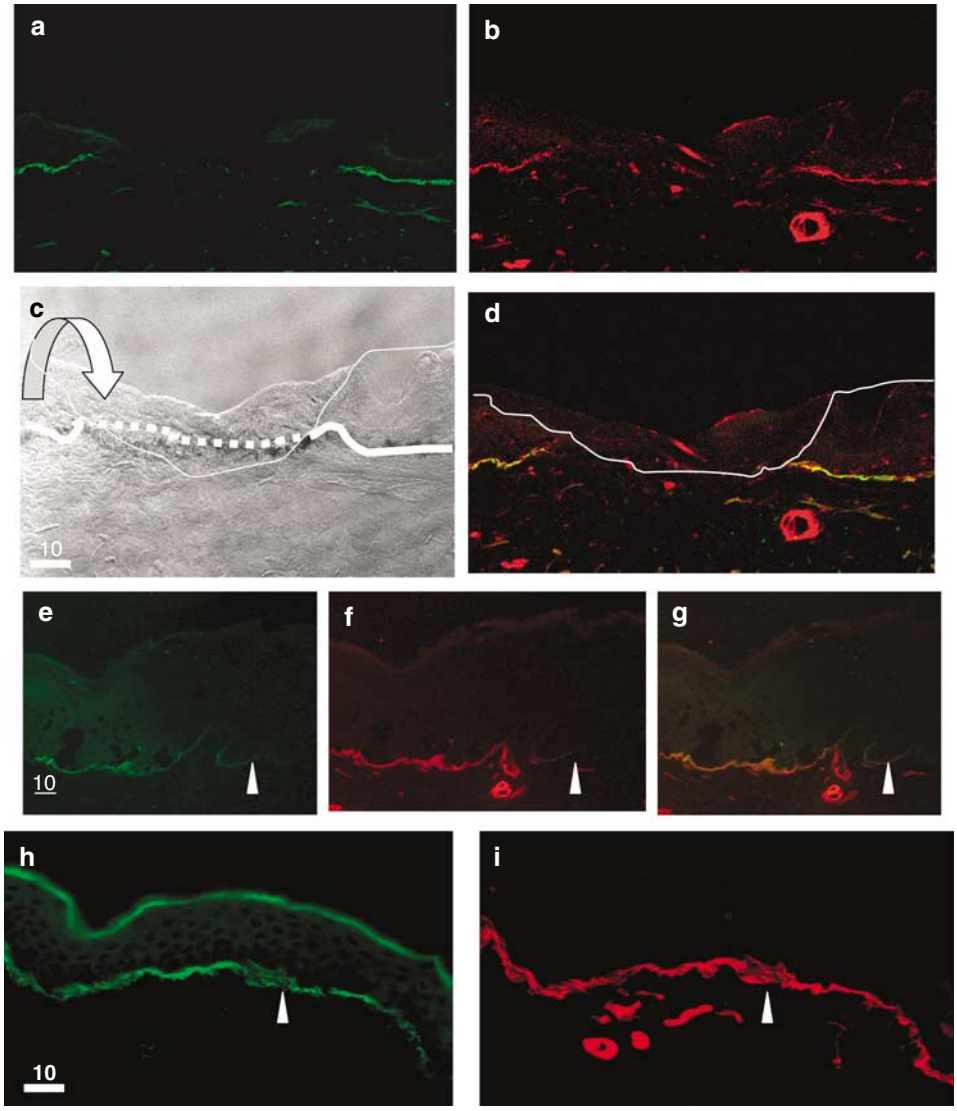

Figure 3 | Technical pitfalls in analysis of α5(IV) staining in the skin. (**a–d**) Folds in the skin (10 × CLSM): apparent focal absence of staining for both (**a**) α5(IV), (**b**) α2(IV) and (**d**) merge along the basement membrane when a folding occurs preventing antibodies penetration (white dotted line **c**). (**e–g**) Air bubble (10 × standard dual immunostaining): segmental absence (arrow) of staining (absence of antibodies penetration) with both α5(IV) (**e**, green) and α2(IV) (**f**, red) antibodies; (**g**) merge. (**h**, **i**) Transversal section of basement membrane (10 × standard dual immunostaining): (**h**) α5(IV) expression appeared decreased because staining was scattered (arrow) when the plane of section was not perpendicular to the BM. The same pattern (**i**) was observed with the α2(IV).

As skin expression of α5(IV) is one of the tools available for genetic counseling, misinterpretation could have disastrous consequences. The use of double standard IF described herein by trained observers should allow more confident interpretation in routine examination.

MATERIALS AND METHODS

We have studied all the skin biopsies addressed to the Department of Pathology during 1 year from 55 patients (35 women and 20 men) suspected of having X-linked AS, based on classical clinical criteria: family history of hematuria with or without progression to renal failure, progressive sensorineural hearing loss; biopsies from five patients investigated for systemic lupus erythematosus served as controls.

Immunohistochemical staining was performed on frozen sections (3 μm) with a commercially available combination of two monoclonal antibodies staining α5(IV) green (fluorescein isothiocyanate; H53 rat IgG2a/kappa and B51 rat IgG2a) and α2(IV) red (texas red; H25 rat IgG1/kappa) (Shigei medical Research Institute: CFT-45325).

The expression of the epidermal basement membrane α5(IV) chain was detected using a direct immunofluorescence method. The sections were air-dried for 30 min, incubated with monoclonal antibodies during 1 h.

The slides were examined with a LEICA DMLB 100 microscope equipped with epifluorescence illumination optics and then with an LSM Pascal confocal laser scanning microscope. The fluorescence intensity of the selected area was quantitatively analyzed using the standard imaging analysis software of an LSM 510 system.

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REFERENCES

1. Hudson BG, Tryggvason K, Sundaramoorthy M *et al.* Alport's syndrome, Goodpastures's syndrome and type IV collagen. *N Engl J Med* 2003; **348**: 2543–2556.
2. Knebelmann B, Breillat C, Forestier L *et al.* Spectrum of mutations in the COL4A5 gene in X-linked Alport syndrome. *Am J Hum Genet* 1996; **59**: 1221–1232.
3. Pescucci C, Mari F, Longo I *et al.* Autosomal dominant Alport syndrome. Natural history of a disease due to COL4A3 or COL4A4 gene. *Kidney Int* 2004; **65**: 1598–1603.
4. Grunfeld JP. Contemporary diagnosis approach in Alport 's syndrome. *Ren Fail* 2000; **22**: 759–763.
5. Kashtan CE. Alport syndrome : is diagnosis only skin-deep ? *Kidney Int* 1999; **55**: 1575–1576.
6. Noël LH. Renal pathology and ultrastructural findings in Alport's syndrome. *Ren Fail* 2000; **22**: 751–758.
7. Jais JP, Knebelmann B, Giatras I *et al.* X-linked Alport syndrome: natural history in 195 families and genotype–phenotype correlations in males. *J Am Soc Nephrol* 2000; **4**: 649–657.
8. Jais JP, Knebelmann B, Giatras I *et al.* X-linked Alport syndrome: natural history and genotype-phenotype correlations in girls and women belonging to 195 families: a 'European Community Alport Syndrome Concerted Action' study. *J Am Soc Nephrol* 2003; **14**: 2603–2610.
9. Van Der Loop F, Monnens LAH, Schröder C H *et al.* Identification of COL4A5 defects in Alport's syndrome by immunohistochemistry of skin. *Kidney Int* 1999; **55**: 1217–1224.
10. Naito I, Nomura S, Inoue S *et al.* X-linked Alport syndrome with normal distribution of collagen IV αchains in epidermal basement membrane. *Contrib Nephrol* 1997; **122**: 134–139.
11. Massella L, Onetti Muda A, Faraggiana T *et al.* Epidermal basement membrane alpha 5(IV) expression in females with Alport syndrome and severity of renal disease. *Kidney Int* 2003; **64**: 1787–1791.
12. Nakanishi K, Iijima K, Kuroda N *et al.* Comparison of a 5(IV) collagen chain expression in skin with disease severity in women with X-linked Alport syndrome. *J Am Soc Nephrol* 1998; **9**: 1433–1440.
13. Gubler MC. Diagnosis of Alport syndrome without biopsy? *Pedriatr nephrol* 2007; **22**: 621–625.
14. Muda AO, Massella L, Giannakakis K *et al.* Confocal microscopy of the skin in the diagnosis of X-linked Alport syndrome. *J Invest Dermatol* 2003; **121**: 208–211.
15. Chuang YH, Dean D, Allen J *et al.* Comparison between the expression of basement membrane zone antigens of interfollicular epidermis and anagen hair follicle using indirect immunofluorescence. *Br J Dermatol* 2003; **149**: 274–281.